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WT1 expression is inversely correlated with *MYCN* amplification or expression and associated with poor survival in non-*MYCN*-amplified neuroblastoma

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ABSTRACT

Neuroblastoma (NB) is the most common extra cranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy. A striking feature of this tumor is its clinical heterogeneity. Several tumor progression markers have been delineated so far, among which *MYCN* amplification, which occurs in about 25% of total NB cases, with the percentage increasing to 30% in advanced stage NB. Although *MYCN* amplification is strongly correlated with NB of poor outcome, the *MYCN* status cannot alone predict all cases of poor survival in NB. Indeed NB without *MYCN* amplification (about 70–80% of NB) are not always favorable. WT1 was initially identified as a tumor suppressor gene involved in the development of a pediatric renal tumor (Wilms' tumor). Here, we describe an inverse correlation between *WT1* expression and *MYCN* amplification and expression. However and most notably, our results show that *WT1* gene expression is associated with a poor outcome for patients showing non-*MYCN*-amplified tumors. Thus *WT1* expression is clinically significant in NB and may be a prognostic marker for better risk stratification and for an optimized therapeutic management of NB.

Abbreviations: NB, neuroblastoma; GGN, ganglioneuroma; WT1, Wilms' tumor protein 1; OS, overall survival.

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1. Introduction

Neuroblastic tumors are thought to arise from neural crest-derived cells that form the developing peripheral sympathetic nervous system. They constitute a wide histological spectrum ranging from ganglioneuroma (GGN), which are benign tumors of fully differentiated cells, to undifferentiated neuroblastoma (NB). NB is the most common and aggressive extra cranial solid tumor of early childhood and is one of the leading causes of cancer death in children between 1 and 4 years. At a biological level, NB tumors are also characterized by a huge heterogeneity. The clinical behavior of neuroblastic tumors ranges from benign forms (spontaneous regression and differentiation) to aggressive malignant disease refractory to all therapies (for review (Brodeur, 2003)). Thus, it is critical to set up a strong risk stratification model that could lead to appropriate treatment, in particular for patients with high-risk neuroblastoma who have a low survival rate.

Genetic alterations account for much of the clinical heterogeneity observed: *MYCN* amplification, somatic and germ line activating mutations in the ALK kinase, deletion within chromosome arms 1p, 11q or 14q, and unbalanced gain of 17q (Maris et al., 2007; Mosse et al., 2009). More recently whole exome and whole genome sequencing analyses have recently identified loss-of function mutations/deletions in chromatin modifiers including *ATRX*, *ARID1A*, and *ARID1B*. However, *ATRX* mutations were more common in patients older than 5 years and no mutations/deletions were identified in the youngest age group (<18 months) (Cheung et al., 2012). Recurrent mutation or focal deletion of *ARID1A* and *ARID1B* were uncovered in 11% of cases in the patient cohort reported by Sausen et al. (2013). The most important alteration is *MYCN* amplification, which occurs in 25% of neuroblastoma and is strongly correlated with advanced disease, drug resistance, and poor outcome (Brodeur, 2003). The presence of amplified *MYCN* copies in tumors is considered as the most powerful genetic marker for prediction of tumor relapse and progression and is associated with a high risk behavior that is taken into account in the treatment strategy independently of disease extension and patient age. It has been shown that the amplification of *MYCN* and the subsequent overexpression of the protein directly contribute to tumorigenesis, as evidenced from the development of tumors in transgenic mouse models (Weiss et al., 1997). MYCN belongs to the Myc family of transcriptional factors. It is predominantly expressed in the developing peripheral neural crest inducing proliferation and migration, with decreased levels associated with terminal differentiation (Wakamatsu et al., 1997). Although *MYCN* amplification is a well-known feature of poor prognosis, it cannot alone predict all poor survival cases. Furthermore, NB cases without *MYCN* amplification are not always favorable (Hiyama et al., 1991). Indeed, it is noteworthy that 70% of high-risk NB cases do not show *MYCN* amplification indicating that other genetic or epigenetic alterations play an important role in tumor aggressiveness and could account for the lower survival of patients (Schwab et al., 2003). Recently, Valentijn et al. identified a set of MYCN-regulated genes that are predictive of poor outcome in patients lacking *MYCN* gene amplification, or with low *MYCN* mRNA levels but with high nuclear MYCN protein levels (Valentijn et al., 2012).

Cellular heterogeneity and level of maturation are hallmarks of human NB tumors and also correlate with clinical behavior (Shimada et al., 2001). Within a single NB, cells from distinct neural crest lineages are present, mainly neuroblastic cells and schwannian stromal cells. The relative abundance of these two lineages has a significant prognosis impact: it has been shown that a tumor with an abundant stromal contingent is frequently associated with a favorable prognosis (Misugi et al., 1985). The Current International Neuroblastoma Pathology Classification (INPC) guidelines use quantification of schwannian stroma to classify these tumors and identify the subset with favorable prognosis (Joshi, 2000). Whether both components share a common neoplastic origin is highly debated (Ambros et al., 2002, 1996; Bourdeaut et al., 2008; Mora et al., 2001; Valent et al., 1999). The same cellular heterogeneity is present in cell lines derived from these tumors. When tumor explants are placed in culture, three phenotypic variants emerged based on their morphological and biological characteristics (Ciccarone et al., 1989): neuroblastic cells or neuronal-like cells (N-type), flat epithelial-like cells or substrate-adherent (also called S- or F-type) and intermediate cells (I-type). The difference in malignant properties between F/S and N cell subtypes may be of clinical relevance. Indeed, in contrast to F/S-type cells, N- and I-type cells are tumorigenic in nude mice, form colonies in soft agar, and possess many similarities with stem cells (Ross et al., 1995). At the transcriptional level, several differences in gene expression were detected including *MYCN*, which is usually expressed at a higher level in N-type cells (Spengler et al., 1997).

The Wilms' tumor gene (*WT1*) was originally identified as a tumor suppressor gene inactivated in Wilms' tumor (nephroblastoma) (Haber et al., 1990). However, recent reports showing that *WT1* is overexpressed and correlated with a bad prognosis in a variety of human cancers including leukemia and breast cancer support a pro-oncogenic function of *WT1* (Inoue et al., 1994; Miyoshi et al., 2002). Recently, in a cohort of 20 NB and 5 GGN, Wang et al. reported that *WT1* expression is higher in mature GGN than in NB and suggested that *WT1* may participate in the maturation of NB (Wang et al., 2011). In the present study, we further explore the functional significance of *WT1* in NB and the impact of its expression on NB patient outcome.

2. Material & methods

2.1. Tumor samples

A total of 67 primary neuroblastic tumors were obtained from patients followed at Gustave Roussy (Villejuif, France) between 1987 and 2009 at the time of diagnosis. Patients' clinical characteristics are shown in Table 1. Diagnosis of NB was determined according to the International Neuroblastoma

Table 1 – Clinical main characteristics of patients.

N°	Patient code	Age at diagnosis (months)	INSS stage	MYCN amplification	Disease-free survival (months)	Overall survival (months)	Present status
1	584	20	4	No	168.5	168.5	A
2	301	49	4	No	18	18	DOD
3	291	92	4	No	150.9	150.9	DOD
4	1415	131	4	No	46.1	46.1	DOD
5	553	109	4	No	80.1	80.1	DOD
6	1271	117	4	No	28	28	DOD
7	1365	37	4	No	142.6	142.6	A
8	1441	77	4	No	28.2	28.2	DOD
9	530	62	4	No	13.1	13.1	A
10	703	42	4	No	136.4	136.4	Al
11	110	58	4	No	22	22	DOD
12	246	61	4	No	44.6	44.6	DOD
13	299	42	4	No	46	46	DOD
14	260	36	4	No	13	13	DOD
15	1741	35	4	No	59	59	A
16	595	54	4	No	42.4	42.4	DOD
17	102	43	4	No	255	255	A
18	1070	43	4	No	27.4	27.4	DOD
19	1146	40	4	No	16.1	19	A
20	2400	85	4	No	20	20	DOD
21	500	32	4	No	165	165	A
22	171	42	4	No	34.1	34.1	DOD
23	1906	29	4	No	18	18	DOD
24	158	54	4	No	39.6	39.6	DOD
25	623	50	4	No	26	26	DOD
26	1736	48	4	No	54	54	A
27	944	43	4	No	22.5	22.5	DOD
28	605	30	4	No	93.7	120.7	A
29	411	73	4	Yes	13	13	DOD
30	872	58	4	Yes	78.4	78.4	DOD
31	1162	23	4	Yes	104	104	A
32	600	20	4	Yes	7	7	DOD
33	105	42	4	Yes	12.6	12.6	DOD
34	358	66	4	Yes	21	21	DOD
35	209	12	4	Yes	15	15	DOD
36	672	60	4	Yes	12	12	DOD
37	307	38	4	Yes	82	82	DOD
38	569	42	4	Yes	120	120	A
39	104	35	4	Yes	5	5	DOD
40	331	19	4	Yes	168	168	A
41	1536	90	4	Yes	5	5	DOD
42	1030	31	4	Yes	104	104	A
43	153	6	1	No	152.8	152.8	A
44	518	13	2	No	112.6	112.6	A
45	613	4	2	No	96	96	A
46	513	40	2	No	79.1	79.1	A
47	1176	7	2	No	99.7	99.7	A
48	471	83	3	No	6.1	6.1	A
49	542	45	3	No	37.4	40.9	A
50	860	5	3	No	120	120	A
51	2446	71	1	Yes	45.7	45.7	A
52	966	2	1	Yes	116.5	116.5	A
53	583	39	2	Yes	20.6	32.56	DOD
54	645	25	2	Yes	7.1	7.1	A
55	2076	7	2	Yes	83.1	83.1	A
56	302	15	3	Yes	29	29	A
57	414	7	3	Yes	145.3	145.3	A
58	499	62	3	Yes	3.4	3.4	DOD
59	2004	1	4	No	24	24	DOD
60	418	4	4	Yes	0.63	0.63	DOD
61	1320	10	4	No	18	18	DOD

(continued on next page)

Table 1 – (*continued*)

N°	Patient code	Age at diagnosis (months)	INSS stage	MYCN amplification	Disease-free survival (months)	Overall survival (months)	Present status
62	1998	2	4	No	10	10	DOD
63	317	7	4	No	40.8	40.8	A
64	400	2	4	No	75.5	75.5	DOD
65	1357	3	4	No	87.9	87.9	A
66	1456	10	4	No	74.8	74.7	A
67	2276	2	4	No	60.2	60.2	A

Disease-free survival was defined as the time from diagnosis to the date of death or the first appearance of relapse. Overall survival was defined as the time from diagnosis to the date of death or last follow-up. DOD: Dead of disease. A: Alive.

Pathologic Classification (INPC) (Shimada et al., 1999) and the stage of disease was determined according to the International Neuroblastoma Staging System (INSS) (Brodeur, 2003). All patients were treated according to the established European protocols. All patients received induction therapy followed by consolidation therapy for high risk NB. Informed consent was obtained from the parents, and the study was approved by a relevant ethics committee. Primary tumor tissues, obtained from patients either by true-cut or after surgery, were immediately snap frozen before being stored in liquid nitrogen until nucleic acid extraction, which was performed as previously described (Gattolliat et al., 2011).

Two publicly available datasets (Tumor Neuroblastoma public-Versteeg-88-MAS5.0-U133p2; accession n° GSE16476, and Tumor Neuroblastoma non-*MYCN* amplified-Seeger-102) were used to analyze the relationship between overall survival and *WT1* expression utilizing the R2 microarray analysis and visualization platform (http://r2.amc.nl). Clinical information on these cohorts were provided in (Molenaar et al., 2012b) and in (Asgharzadeh et al., 2006), respectively. In brief, the first dataset contains 88 primary neuroblastoma tumors of all stages (stage 1, n = 8; stage 2, n = 15, stage 3, n = 13 with 1 *MYCN*-amplified, stage 4, n = 40 with 15 *MYCN*-amplified, stage 4S, n = 12). The tumors used in this dataset have been profiled utilizing the Affymetrix U133plus2.0 genechip (Affymetrix, Santa Clara, CA, USA) (Molenaar et al., 2012a; Valentijn et al., 2012). The second dataset contains 102 untreated primary neuroblastomas without *MYCN* gene amplification obtained from children whose ages at diagnosis ranged from 0.1 to 151 months. Affymetrix microarrays were used to determine the gene expression profiles (Asgharzadeh et al., 2006).

2.2. Cell lines

The well-characterized human NB cell lines used in this study displayed either *MYCN* amplification (LAN-1, IGR-N-91, IMR-32, IGR-N-835, NBL-w-N, NBL-w-S, SK-N-BE(2)), non-*MYCN*-amplification (SK-N-SH, SK-N-AS, SH-EP1, SH-SY-5Y), or *MYCN* conditional expression (SK-N-AS/*MYCN*-ER and SH-EPTet21N). Cells were obtained from ATCC (SK-N-AS, IMR-32, SH-SY-5Y, SK-N-BE(2)), from Dr J. Bénard (Gustave Roussy, LAN-1, IGR-N-91, IGR-N-835, NBL-w-N, NBL-w-S), from Dr I. Janoueix-Lerosey (Institut Curie, SK-N-SH, SJNB-1, SK-N-FI, GIMEN), and from Dr L. Valentijn (Department of Human Genetics, Academic Medical Center, Amsterdam, for SK-N-AS/*MYCN*-ER, SH-EP1 and SH-EPTet21N). All cell lines were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), L-glutamine (2 mM) and non-essential amino acids (PAA). They were confirmed negative for mycoplasma by routine testing. All cell lines were characterized by short tandem repeat analysis (STR) using the Promega powerplex 21 PCR kit (Eurofins). The STR profiles of SK-N-AS, IMR-32, SH-SY-5Y, SK-N-BE(2), LAN-1, SK-N-SH, SK-N-FI, and GIMEN matched with the existing on-line DSMZ database (http://www.dsmz.de/de/service/service-human-and-animal-cell-lines/online-str-analysis.html). As expected, NBL-w-N and NBL-w-S showed identical profiles. IGR-N-91, IGR6N-835 and SJNB-1 cell lines were not present in DSMZ or ATCC STR database. All cell lines were also systematically validated at reception and during usage by checking for *MYCN* expression and amplification by western blot and real-time quantitative PCR, respectively. In SK-N-AS/*MYCN*-ER cells, *MYCN* translocation into the nucleus was obtained upon addition of 50 nM 4-hydroxytamoxifen (OHT, Sigma) (Valentijn et al., 2005). The SHEPTet21N *MYCN* expression system previously described (Kramps et al., 2004) was used to conditionally express *MYCN* in a non-*MYCN*-amplified background. *MYCN* expression was switched off by the addition of 10 ng/ml of tetracycline to growth media.

2.3. Lentiviral cell infections and transient transfection experiments

Lentiviral particles (pLKO.1/shRNA/WT1) targeting *WT1* expression and control vectors were purchased from Sigma (MISSION® pLKO.1-puro shRNA WT1 and Non-Mammalian shRNA Control). SH-EP1 and SK-N-AS were transduced with lentiviral particles in the presence of proteamine sulfate (5 μg/mL) at a multiplicity of infection of approximately 0.5–1.0. Four days post-infection, the cells were washed and selected for 3 days with puromycin (Sigma) at 1 μg/mL. LAN-1 NB cells were transiently transfected with two human WT1 isoforms WT1 (+17AA/−KTS) and WT1 (−17AA/−KTS) cloned into pcDNA3, and the empty vector as a control (Tajinda et al., 1999). Both WT1 isoforms are characterized by the absence of three amino acids, KTS, between zinc fingers III and IV. They differ by the presence (+) or the absence (−) of exon 5, which encodes 17 amino acids. Briefly, 5×10^5 cells were seeded in a 6-well plate. Twenty four hours after plating,

cells were transfected using X-tremeGENE HP DNA transfection reagent according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) with 2 μg per well of the plasmids. Twenty four hours after the transfections, the cells collected for total RNA and proteins were extracted as described below.

2.4. Quantitative reverse transcription PCR

Total cellular RNA was extracted using TRIzol reagent (invitrogen). Utilizing the "transcriptor first Strand cDNA Synthesis" Kit (Roche Diagnostics), an RT reaction was carried out from 1 μg mRNA (for cell lines) and 250 ng (for tumor samples) with oligo(dT)$_8$ primers for cell line RNAs and Random Hexamer for tumor sample RNAs. The mRNA expression was quantified by quantitative reverse transcription-PCR (qRT-PCR) using LightCycler technology. The *WT1* mRNA was quantified using the "Light Cycler FastStart DNA MasterPLUS SYBR Green" Kit (Roche Diagnostics) according to the manufacturer's instructions. Quantitative *WT1* mRNA levels were normalized to the expression of *GAPDH*. Primer sequences were the following: *WT1* Forward primer: 5′-GGGTACGAGAGCGATAACCA-3′ and *WT1* Reverse primer: 5′-ATGCCGACCGTACAAGAGTC-3′; *GAPDH* Forward primer: 5′-CACC CATGGCAAATTCCATGGC-3′ and *GAPDH* Reverse primer 5′-GCATTGCTGATGATCTTGAGGCT-3′. The *MYCN* mRNA expression was quantified using the "FastStart Universal Probe Master Mix" Kit (Roche Diagnostics) on the StepOnePlus RealTime PCR System (Applied Biosystems) according to the MIQE Guidelines (Bustin et al., 2009). The relative expression was calculated based on the comparative $2^{-\Delta\Delta CT}$ quantification method (Livak and Schmittgen, 2001). The *TFRC1* gene was used as a normalizer (Taqman Gene Assays: *TFRC1* hs00174609; *MYCN* hs00232074).

2.5. Quantification of MYCN copy number

The *MYCN* copy number was determined from purified genomic DNA (20 ng) using (i) the Taqman *MYCN* Copy Number Assay (4400292, Applied Biosystems), (ii) the Taqman RNase P Copy Number Reference Assay (4403326, Applied Biosystems), and (iii) the FastStart Universal Probe Master (ROX, Roche Diagnostics). Reactions were run on a 96-well RealTime StepOnePlus PCR System (Applied Biosystems) and amplified at 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The number of copies of the target sequence in each sample was determined by relative quantification using the comparative $2^{-\Delta\Delta CT}$ quantification method.

2.6. Western blot analysis

Total cellular proteins were extracted with SDS lysis buffer (SDS 8%, Tris–HCl 250 mM pH 6.8, 2.5 mM NaF, 0.2 mM sodium orthovanadate, protease inhibitor cocktail from Sigma), separated by SDS-PAGE (10%), and transferred onto a PVDF membrane. After saturation, blots were probed with mouse monoclonal primary antibodies: anti-N-Myc (sc-56729, Santa Cruz); anti-NSE (clone VI-H14, Dako); anti-WT1 (clone 6F-12, Dako); anti-Vimentin (clone V9, Thermoscientific); anti-Neurofilament (clone 2F11, Thermoscientific). After washing, blots were incubated with horseradish peroxydase conjugated anti-mouse IgG antibodies and then developed by enhanced chemiluminescence (Perkin Elmer, Life sciences). Rabbit polyclonal HRP-conjugated anti-GAPDH (ab 9385, Abcam) was used as a control for equal loading.

2.7. Statistical analyses

The qRT-PCR data were analyzed using GraphPad Prism software to generate scatter plots and two-tailed non parametric tests (Mann–Whitney U-test and Spearman test) were used to compare the different neuroblastoma groups. To generate Kaplan–Meier survival curves, we converted the *WT1* expression levels measured by qRT-PCR to a dichotomous variable, using the median of the expression level as a cut-off value. This procedure enabled the partition of samples into two level groups with high WT1 expression >9.75 and low WT1 expression ≤9.75. The non-parametric Kaplan–Meier estimates for overall survival (OS) under the two conditions were compared by log-rank test. Kaplan–Meier analysis and comparison of WT1 expression between different patient subgroups for the publicly available neuroblastoma dataset from patient cohorts were performed online on the R2 platform (http://r2.amc.nl) and the resulting curves and p-values (log-rank test) were downloaded.

Finally, a multivariate analysis used the Cox proportional hazards regression method to investigate the independent prognostic power of *WT1* when other variables (age, disease stage, and *MYCN* status) were simultaneously considered. Since a relatively small number of patients ≤18 months (n = 19) were involved in the study compared to patients >18 months (n = 48), the median age (37 months) was considered to dichotomize the patients by age. Disease stage was dichotomized with respect to the stage of metastatic progression (*i.e.*, stage 1, 2, and 3 *vs* stage 4). As for the *WT1* gene, the median value of its expression in the cohort was used as described above to dichotomize the patients into two level groups (high and low *WT1*).

3. Results

3.1. WT1 expression is inversely correlated with MYCN amplification and/or expression in neuroblastoma tumors and cell lines

In order to gain insight into the role of WT1 in NB, we analyzed *WT1* mRNA levels in a cohort of 67 primary neuroblastoma tumors (Figure 1A). We found no significant correlation between *WT1* mRNA levels and *MYCN* amplification status in the overall cohort (p = 0.087). However considering only stage 4 tumors (n = 51), there was a significantly higher *WT1* expression (p = 0.005) in non-*MYCN*-amplified tumors (n = 35) than in those exhibiting *MYCN* amplification (n = 16). The statistical significance of this result was further enhanced when the cohort was limited to high-risk NB of stage 4 in children over 18 months (p = 0.0024). Importantly and as shown by others, we found that high *MYCN* mRNA levels were highly correlated with *MYCN* gene amplification (p = 0.0006, Supplementary

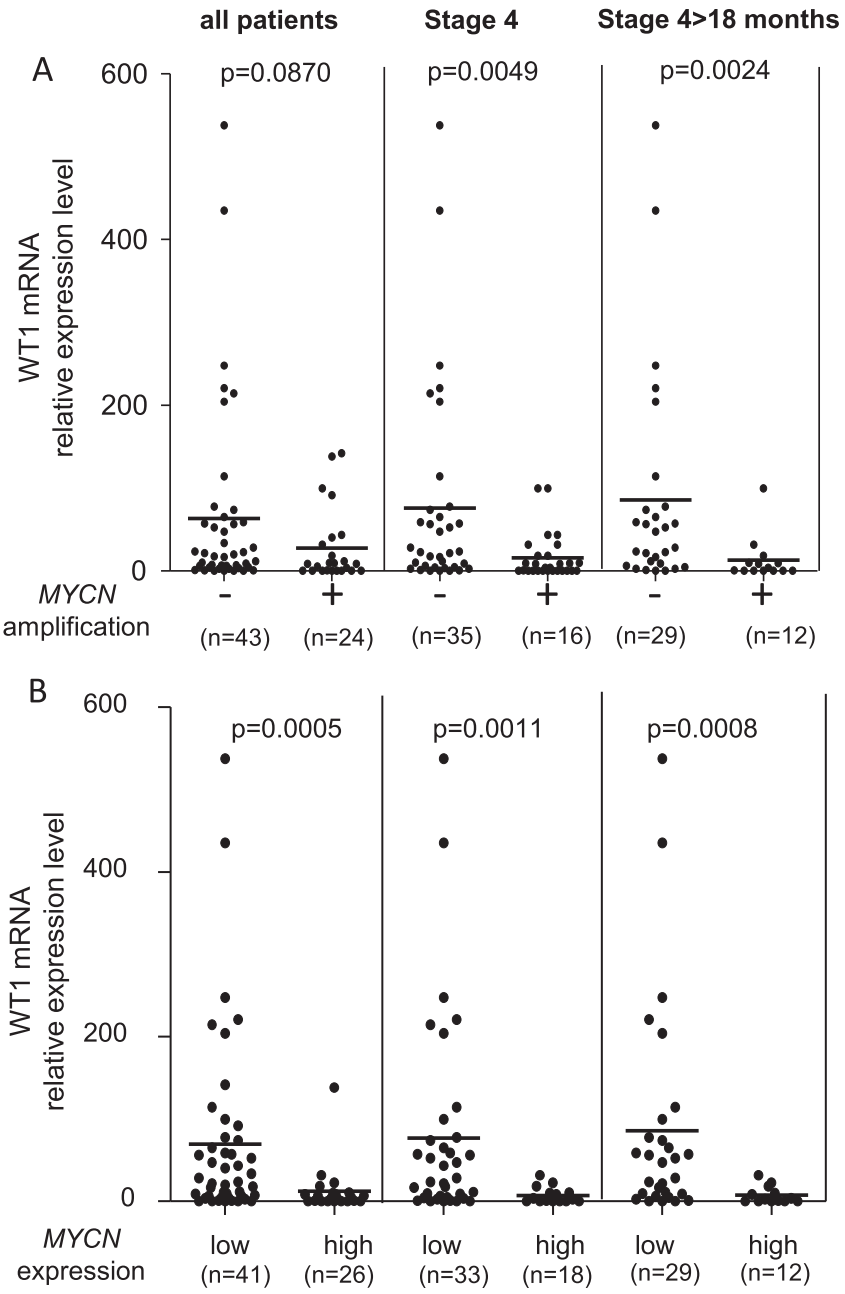

Figure 1 – Scatter plots showing mean of *WT1* mRNA expression versus *MYCN* amplification (A) or expression (B) in Neuroblastoma. The relative *WT1* mRNA expression, *MYCN* copy number and relative *MYCN* mRNA expression was monitored by qPCR and RT-qPCR, respectively, in a set of 67 primary neuroblastoma, a subset of 51 primary stage 4 neuroblastoma, and a subset of 43 primary stage 4 neuroblastoma in children over 18 months. Expression values were normalized to *GAPDH* housekeeping gene. Two-tailed Mann–Whitney tests were used. An expression cut-off value of 0.05 was used to dichotomize the patients into two groups (high *MYCN* expression > 0.05; low *MYCN* expression ≤0.05).

Figure S1). We then examined *WT1* and *MYCN* mRNA levels in the whole cohort (n = 67). A negative correlation between *WT1* and *MYCN* expressions was found (p = 0.0029). An optimal *MYCN* expression cut-off value of 0.05 was used to dichotomize the study cohort into low and high *MYCN* expressions. *WT1* mRNA levels were significantly higher in tumors with low *MYCN* expression than in those with high *MYCN* expression (Figure 1B) within each patient group (p = 0.0005, 0.0011 and 0.0008 in the whole study cohort, stage 4 tumors, and high-risk NB of stage 4 in children over 18 months, respectively).

We then examined the relationship between *MYCN* gene amplification status, *MYCN* expression and *WT1* expression in 14 neuroblastoma-derived cell lines (Figure 2). All 7 NB

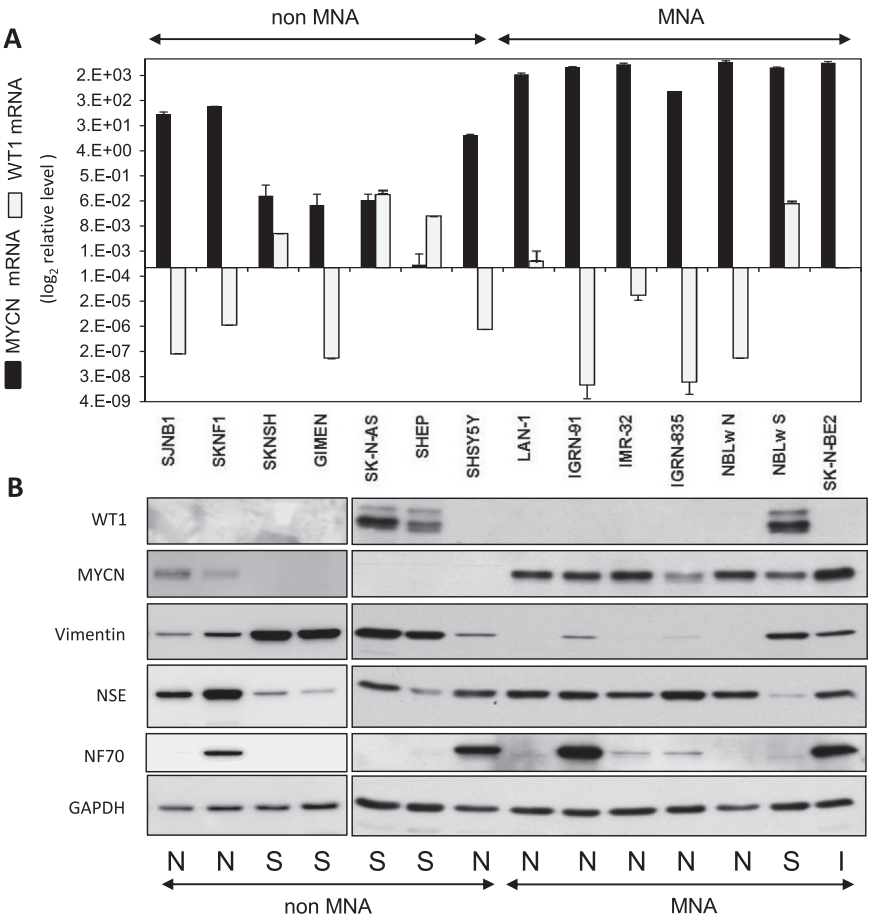

Figure 2 – WT1 expression is inversely correlated to *MYCN* amplification and expression in NB cell lines. A. Expression profiles of *WT1* and *MYCN* in neuroblastoma cell lines measured by quantitative PCR. Expression values were normalized to *GAPDH* housekeeping gene. Error bars correspond to S.E.M. B. Whole cell extracts of NB cells were separated by SDS-PAGE and probed with anti-WT1, MYCN, Vimentin, NSE and NF70 antibodies. GAPDH was used as a loading control. MNA: *MYCN*-amplified; non MNA: *MYCN*-non-amplified; S: substrate adherent type (F/S); N: neuronal type; I: intermediate type.

exhibiting *MYCN* amplification expressed high levels of MYCN protein. Of note 2 NB with non-amplified *MYCN*, SJNB-1 and SK-N-FI, expressed detectable levels of *MYCN* at both mRNA and protein levels. Interestingly, none of the 9 NB expressing MYCN protein but one (NBL-w-S) expressed detectable levels of *WT1* mRNA or protein. Altogether, these observations corroborate the data obtained in patients and suggest an inverse correlation between *WT1* and *MYCN* expressions in neuroblastic tumors as well as in neuroblastoma cell lines.

3.2. *F/S-type neuroblastoma cells express high levels of WT1*

So far, by analogy with what is observed in NB tumors, at least three cellular phenotypes were identified in neuroblastoma cell lines: N-type neuroblastic cells, F/S-type flat substrate-adherent cells that can be distinguished by morphologic features and biochemical markers, and intermediate I-type cells, which represent an intermediate stage between F/S- and N-type cells in terms of morphology and biochemical markers. The cell lines selected in this study differ not only with respect to *MYCN* amplification but also to their phenotypes, which can be defined by the expression of specific F/S- or N-type markers. Indeed, as shown in Figure 2B, Vimentin (a F/S-type associated marker) was expressed at a higher level in F/S-type cells (SK-N-SH, GIMEN, SK-N-AS, SH-EP1, and NBL-w-S), whereas NSE (N-type associated marker) was expressed at a higher level in N-type cells (SJNB-1, SK-N-FI SH-SY-5Y, LAN-1, IGR-N-91, IMR-32, IGR-N-835, and NBL-w-N). SK-N-BE2 cells, which are of I-type express detectable levels of all three N-type associated markers (Vimentin, NSE and NF70).

Importantly, the human neuroblastoma cell lines SH-EP1 and SH-SY-5Y are phenotypically distinct subclones derived from the same neuroblastoma SK-N-SH cell line that contains no *MYCN* amplification (Biedler et al., 1973). SH-SY-5Y represents a clonal population of N-type cells, whereas SH-EP1 is one F/S-type cell line derived from the same parent line. In a similar way, the two *MYCN* amplified NBL-w-S and NBL-w-N cell lines are the respective F/S and N-type subclones of the NBL-W neuroblastoma parental cell line. Looking at the expression of WT1 with both quantitative RT-PCR (Figure 2A)

and western blot (Figure 2B), we observed that the F/S-type cell lines, SH-EP1 and NBL-w-S, expressed high levels of WT1, whereas this protein was not detected in their N-type respective counterparts, SH-SY-5Y and NBL-w-N cells. Note that SK-N-AS, another S-type cell line, also exhibited high levels of WT1 protein. Taking advantage of the IGR-N-91 *MYCN* amplified neuroblastoma cell line showing N-type and the previously described possibility to switch them to F/S phenotype by the stable expression of a dominant negative (DN) variant of the catalytic subunit of telomerase (hTERT) (Samy et al., 2012), we showed that this shift appeared to be associated with higher WT1 protein levels (Supplementary Figure S2). This observation corroborates the above results suggesting that the high expression of WT1 appears mainly associated with F/S-type NB cells. Of note, although GIMEN expressed very low levels of MYCN and exhibited features of F/S phenotype, WT1 was not detected either at mRNA or protein levels.

3.3. *Ectopic overexpression and siRNA mediated repression of* WT1 *do not influence the expression of* MYCN

Previous studies showed that the WT1 protein can bind to the *MYCN* promoter and repress its transcriptional activity (Zhang et al., 1999). WT1 exist in different isoforms due to alternative pre-mRNA splicing. DNA binding specificity is determined by insertion or removal of three amino acids between zinc finger III and IV (referred to as WT1(+KTS) and WT1(−KTS)). The −KTS isoform have been reported to activate or repress target genes including *MYCN* (Zhang et al., 1999). The inverse relationship observed above between *MYCN* and *WT1* expressions led us to investigate in detail whether there was a direct linkage between *MYCN* and *WT1* expressions. To address this question we first examined the effect of ectopic expression of two WT1(−KTS) isoforms in the LAN-1 NB cell line expressing constitutively very low level of *WT1* mRNA and undetectable level of endogenous WT1 protein. The transient expression of both isoforms of WT1 was confirmed by western blot (Figure 3A). However, this ectopic expression did not modify MYCN level either at the protein (Figure 3A) or RNA levels (Supplemental Figure S3A).

We then examined the effect of downregulating *WT1* expression. Two *MYCN* non-amplified cell lines (SHEP-1 and SK-N-AS) were transduced with a shRNA containing lentiviral vector, constitutively producing siRNA against *WT1*. Stable transfected cells were selected with puromycin and kept in culture for several passages without modifications of cell proliferation rate. The silencing was confirmed by western blot analysis (Figure 3B). However, the data show that *WT1* silencing did not affect *MYCN* expression either at mRNA level (Supplemental Figure S3B) or at protein level (Figure 3B). This result apparently rules out a direct role for *WT1* in *MYCN* expression.

3.4. WT1 *expression is not directly regulated by* MYCN

To further understand the relationship between *MYCN* and *WT1* in neuroblastoma, we then used two functionally inducible *MYCN* systems in which *MYCN* activities can be switched on or off. The first cell line, SK-N-AS/*MYCN*-ER, is a *MYCN* single copy cell line, which constitutively expressed a hybrid of *MYCN* – estrogen receptor (ER) protein that can be activated

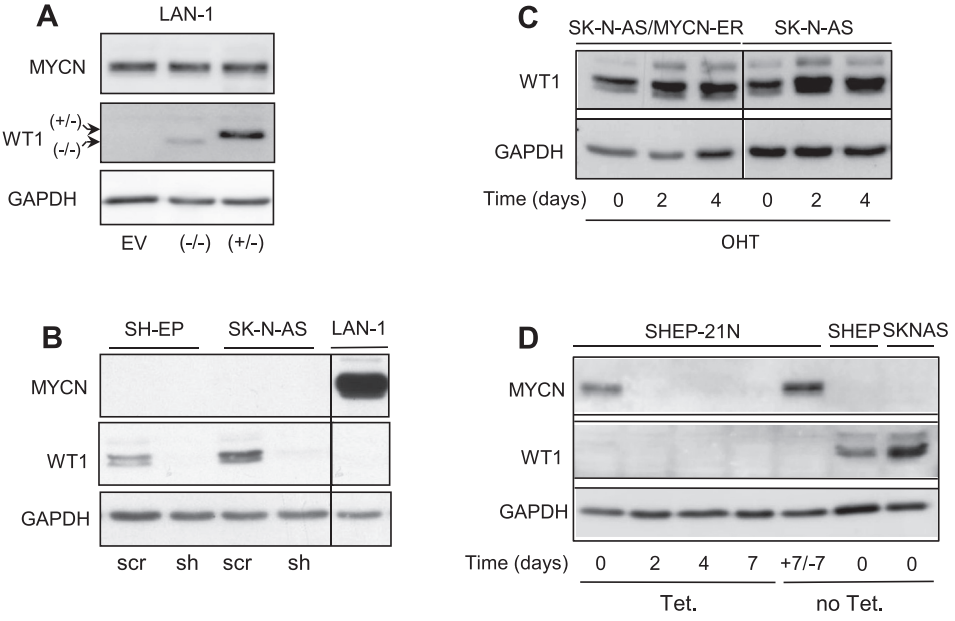

Figure 3 – Mechanistic analysis of the link between *WT1* and *MYCN*. A. LAN-1 NB cells were transiently transfected with two human WT1: WT1 (+17AA/−KTS) and WT1 (−17AA/−KTS), and the empty vector as a control (EV). B. Endogenous WT1 expression was targeted by transduction of a shRNA against *WT1* (sh) or a scrambled control RNA (scr). C. *MYCN* was activated in SK-N-AS/MYCN-ER cells by the addition of 4-OHT for the indicated time. D. *MYCN* expression was switched off for 7 days by tetracycline treatment (+tet) in SH-EP21N cells, a subclone of the *MYCN* single copy SHEP cell line transfected with a tetracycline regulated *MYCN* expression vector. Subsequently *MYCN* was re-expressed by removal of tetracycline (no tet) for 7 days (+7/−7). WT1 and MYCN protein levels were determined by western blot. Loading of equal protein amounts was assessed by the detection of GAPDH.

only after ER ligand binding. The addition of 4-hydroxytamoxifen (4-OHT) to the culture media resulted in the rapid translocation of the chimera protein to the nucleus and induction of *MYCN* target genes such as hTERT (Eberhardy et al., 2000). We therefore activated MYCN in SK-N-AS/*MYCN*-ER cells with 4-OHT and analyzed *hTERT* mRNA levels and *WT1* expression at both protein and mRNA levels (Figure 3C and Supplementary Figure S3C, *upper panel*). Activation of *MYCN* in SK-N-AS/*MYCN*-ER caused more than a 4-fold increase in *hTERT* mRNA signal (Supplementary Figure S3, *upper panel*) confirming that the experimental system is working efficiently as expected. However, we did not find any decrease in *WT1* mRNA (Supplemental Figure S3C, *lower panel*) or protein level (Figure 3C) in the induced SK-N-AS/*MYCN*-ER cells compared to the SK-N-AS parental cells. We then used SHEP-21N neuroblastoma cells, which derive from the *MYCN* single copy SHEP cell line transfected with a tetracycline regulated (tet-off) *MYCN* expression vector, and therefore express high levels of MYCN protein (Lutz et al., 1996). In the absence of tetracycline, these cells expressed high levels of MYCN and undetectable levels of WT1 compared to the SHEP parental cells that were negative for MYCN expression but expressed high levels of WT1 (Figure 3D). This observation supports the inverse relationship demonstrated between *WT1* and *MYCN* expressions. Treatment of SHEP-21N cells with tetracycline resulted in a loss of MYCN protein expression and tetracycline removal resulted in the re-expression of MYCN. However, the complete repression of *MYCN* expression in SHEP-21N cells did not reactivate *WT1* expression. These results demonstrate that a rapid modulation of *MYCN* expression does not impact *WT1* expression. Altogether these observations support a link between *WT1* and *MYCN* but not a direct transcriptional effect of *MYCN* on *WT1*.

3.5. *High expression of* WT1 *is associated with lower survival in non* MYCN *amplified NB*

To determine whether *WT1* expression has any influence on the survival of patients with NB, the median value of WT1 expression in the whole cohort was used to dichotomize the patients into two groups (low *WT1* expression ≤9.75 *vs* high *WT1* expression >9.75). The overall survival of patients with high *WT1* expression was then compared with the survival of patients showing low *WT1* expression. As presented in the Kaplan–Meier curve of Figure 4, patients with high *WT1* expression had a poorer survival than patients with low *WT1* expression ($p = 0.0344$, log-rank). We then subdivided the expression data into *MYCN*-amplified and non-*MYCN*-amplified groups to separately evaluate the prognostic relevance of *WT1* in these two groups (Figure 4). Notably, NB patients without *MYCN* amplification had a statistically significant poorer survival when *WT1* expression was high ($p = 0.0157$) suggesting that *WT1* expression is a relevant prognostic marker in this particular subset of patients. In contrast, even though short-term survival appeared to be higher in the low-expression *WT1* subset, *WT1* expression levels had no significant impact on the long-term survival of NB patients with *MYCN* amplification ($p = 0.372$). Mining publicly available clinical neuroblastoma expression datasets [R2: microarray analysis and visualization platform (http://r2.amc.nl)] further confirmed these observations. Indeed, a first dataset (Tumor Neuroblastoma public-Versteeg-88-MAS5.0-U133p2 dataset) including expression data from 88 tumor samples of untreated patients along with survival outcome data showed that high *WT1* expression is significantly associated with patient risk of death in non-*MYCN*-amplified tumors (Supplementary Figure S4A). This is consistent with the results of a second dataset (Tumor Neuroblastoma non MYCN amplified-Seeger-102-MAS5.0-U133a) including expression data from 102 tumor samples of MYCN non-amplified untreated patients (Supplementary Figure S4B).

Taken together, these results demonstrate that *WT1* transcript levels discriminate non-amplified-*MYCN* NB patients with favorable and unfavorable outcomes. In addition, though the size of the patient cohort was relatively small, the multivariate Cox regression models based on OS, integrating established risk markers (*MYCN* status, tumor INSS stage and patient age at diagnosis) determined *WT1*

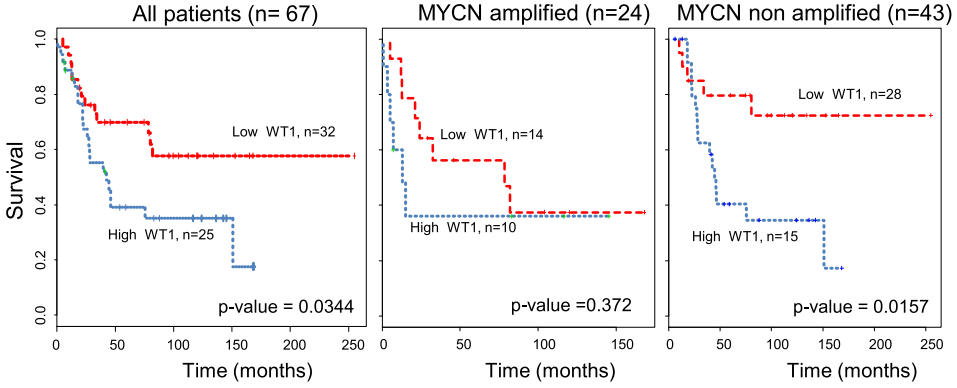

Figure 4 – High *WT1* expression is significantly associated with lower patient survival in non-*MYCN*-amplified neuroblastoma. Kaplan–Meier curves of overall survival for low *vs* high *WT1* expression in the entire cohort (67 patients, right panel), in 24 *MYCN* amplified (middle panel) and 43 *MYCN* non-amplified patients (left panel). The cohort of NB patients was dichotomized using the median value of *WT1* expression (9.75) into two classes: high (*WT1* expression >9.75) and low (*WT1* expression ≤9.75). The log-rank test was used to assess differences in survival of the neuroblastoma subsets indicated. The association of high *WT1* expression with poor prognosis is statistically significant ($p = 0.0157$) only in *MYCN* non-amplified NB.

expression as a significant independent prognostic marker for OS (Table 2).

4. Discussion

There is increasing evidence that analysis of *MYCN* gene amplification alone does not ensure completely accurate prognostic grouping. Therefore, the discovery of prognostic factors can help to stratify patients and provide optimized treatment. A recent report identified a 157-gene signature in very poor prognosis NB tumors as more powerful than *MYCN* amplification or *MYCN* expression alone (Valentijn et al., 2012). Most of these tumors, although having low *MYCN* mRNA levels, displayed high levels of MYCN protein. The Wilms' tumor gene (*WT1*) was initially identified as a tumor suppressor gene involved in the development of Wilms' tumor. A recent study identified WT1 as a potential factor participating in cell suppression of proliferation and the maturation of neuroblastoma (Wang et al., 2011). Here, we show using primary neuroblastoma tumors that *WT1* expression inversely correlates with *MYCN* expression. Even though we have been able to reproduce this observation on neuroblastoma cell lines, using various distinct *MYCN* inducible cell models and *WT1* silenced cells, it was not possible to uncover any mechanistic link between *WT1* and *MYCN* expressions. One possible explanation is that the loss of *WT1* could have been selected for during tumor outgrowth in cells showing high levels of *MYCN*, which would explain why *WT1* was not recovered in the *MYCN*-157 gene signature (Valentijn et al., 2012).

Recent reports showed *WT1* expression in the majority of Schwann cells of fetal nerve fibers and in the sympathetic neuroblasts of the human peripheral sympathetic nervous system. WT1 progressively disappears with differentiation. Its expression in Schwann cells could underline its involvement in tumorigenesis by maintaining these cells in a more undifferentiated state (Parenti et al., 2014, 2015). This idea is further supported by a recent report showing cytoplasmic WT1 immunostaining in Schwann cells of the stroma component of immature ganglioneuroblastoma (Salvatorelli et al., 2015). Therefore this expression could be associated to a given state of cell differentiation as suggested by Wang et al. (2011). Even though in NB cell lines the flat and substrate-adherent F/S-type cells expressing stromal cell markers may differ from Schwann cells in actual *in vivo* NB tumors (Ambros and Ambros, 1995; Biedler et al., 1973; Ambros et al., 2002, 1996; Bourdeaut et al., 2008; Mora et al., 2001; Valent et al., 1999), and the sample size was rather small, it is striking to note that WT1 was never detected in N-type neuroblastoma cell lines, whereas NB cells expressing WT1 are all of the F/S-type. It was already shown that whereas N- and I-type NB cells have abundant *MYCN* mRNA and protein, in most F/S cells, *MYCN* expression is downregulated (Ambros et al., 1997; Narath et al., 2007). These observations fit to the present data obtained on NB cell lines showing a relationship between *MYCN* amplification/expression, WT1 expression and the cell phenotype. Interestingly, some reports hypothesized that *MYCN* downregulation in F/S-type NB cells could be associated with a revertant phenotype characterized by changes in the expression pattern, a reduction of proliferation rate and an increasing capacity to enter a senescence program (Ambros et al., 1997). WT1 was originally identified as a tumor suppressor in Wilms tumor (Haber et al., 1990). However, it has been shown to have either tumor suppressive or oncogenic functions in a manner that appears to be cell-type and context-dependent (Yang et al., 2007). Supporting this idea, in a RNAi-based negative selection screen, the loss of WT1 was shown to inhibit cell proliferation and activate a senescent program specifically in cells expressing oncogenic KRAS but not in cells expressing wild-type KRAS (Vicent et al., 2010). In the present study, *WT1* silencing in two F/S-type NB cell lines did not affect cell proliferation rate nor induce signs of senescence. Therefore, the functions of WT1 in these cells and whether these observations made in a panel of NB cell lines could be relevant in tumors and related to the outcome of the disease remain to be determined.

A previous survival analysis performed on a cohort of 27 NB patients indicated that the expression levels of *WT1* did not affect prognosis (Wang et al., 2011). However, in this study, the *MYCN* status of patients was not taken into account. In the present study, the overall survival analysis conducted on a cohort of 67 NB patients suggests that a high level of *WT1* expression is significantly associated with reduced overall survival only in patients without *MYCN* amplification. Importantly, this finding was clearly confirmed in the Versteeg microarray dataset. This observation suggests that by investigating *WT1* expression in relation with *MYCN* status, it is possible to ascribe prognostic significance to *WT1* expression in non *MYCN*-amplified NB.

Recent studies have shown that *WT1* is often overexpressed in leukemia and various types of solid tumors, this expression being significantly associated with poor prognosis (Inoue et al., 1994; Miyoshi et al., 2002; Tamaki et al., 1996). Thus, *WT1* is now regarded as a molecular target for immunotherapy in these cancers. Clinical trials of WT1-targeted immunotherapy have confirmed its safety and clinical efficacy (Sugiyama, 2010). Recent reports suggest that WT1-based immunotherapy should be a promising alternative for high-risk solid tumors in childhood (Hashii et al., 2010; Ohta

Table 2 – Multivariate Cox regression analysis of overall survival in NB patients (N = 67) considering age at diagnosis, INSS stage, *MYCN* status and *WT1* expression.

Marker (Patients N = 67)	Hazard ratio (95% CI)	p-Value
Diagnosis age (>median *vs* ≤median)[a]	2.21 (1.02–4.78)	0.0447
INSS stage (1, 2, 3 *vs* 4)	7.44 (1.56–35.48)	0.0117
MYCN status (amplified *vs* not amplified)	4.25 (1.58–11.43)	0.0042
WT1 expression (high *vs* low)[b]	3.09 (1.18–8.13)	0.0220

a Because of the small number of patients ≤18 months involved in the study (n = 19) compared to the number of patients >18 months (n = 48), we considered the median value (37 months) to dichotomize patients by age.
b For *WT1* gene, the median value of its expression in the cohort was used to dichotomize the patients into two groups (high *WT1* expression >9.75 and low *WT1* expression ≤9.75).

et al., 2009). Therefore, our observations, if confirmed in larger groups of NB, support the case for using of *WT1* as a potentially prognostic factor of great interest in tumors without *MYCN* amplification, which should be taken in consideration for adapting the therapeutic strategy. Further studies should therefore be performed to explore whether *WT1*-based therapeutic approaches could be effective in these specific types of non-*MYCN*-amplified NB tumors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.09.010.

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